ADHERENT Ia⁺ MURINE TUMOR LINES
WITH CHARACTERISTICS OF DENDRITIC CELLS

I. Morphology, Surface Phenotype, and Induction
of Syngeneic Mixed Lymphocyte Reactions*

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Much evidence has demonstrated that antigen-induced proliferation of T lymphocytes is under control of the I region of the H-2 gene complex and appears to be expressed at the level of an adherent macrophage that expresses membrane Ia antigens (1). Additional Ia-bearing accessory cells such as Langerhans' cells (2) in the basal layer of the epidermis have been identified. These cell types have been shown to bind antigen and present it to T cells and/or to trigger T cell proliferation in a mixed leukocyte reaction (3).

An Ia⁺ dendritic cell (DC)[1] has also been identified in the spleen of mice (4) and has been shown to possess accessory cell functions for the inductive phase of certain immune responses in vitro (5), including the induction of syngeneic T lymphocyte proliferation (6), enhancement of T cell proliferation during periodate-induced mitogenesis (7), enhancement of cytolytic T cell induction toward trinitrophenyl-modified syngeneic target cells (8), and augmentation of antigen-induced proliferation of primed T cells (9). Many of these accessory cell functions are thought to be regulated by the expression of surface Ia molecules on DC (5). Extensive work by Steinman and colleagues (4–6) has clearly identified DC as being distinct from splenic macrophages (MΦ). These loosely adherent cells are characterized by numerous dendritic processes, a preponderance of rod-shaped mitochondria in the cytoplasm, low endocytic activity, and an absence of Fc and C3b receptors (5). Procedures for the isolation of enriched fractions of DC have been described, but because DC represent a minor fraction in the spleen, the obtainable cell yield is small.

Utilization of Ia⁺ continuous cell lines as accessory cells for studying the inductive phase of the immune response would be advantageous because of the homogeneity of cell lines and the availability of large numbers of cells for biochemical analysis. However, adherent Ia⁺ cell lines that express accessory cell function have not been

available. We describe in this report the isolation and characterization of several adherent cell lines, cloned from murine P388 leukemia cells, which possess many morphological and functional similarities to DC of the spleen, including dendritic processes, large numbers of mitochondria, surface expression of Ia antigens, and induction of syngeneic and allogeneic mixed lymphocyte reactions.

Materials and Methods

Isolation of Cell Lines. The P388 leukemia cell line, which was routinely maintained by weekly passage in vivo in DBA/2 mice, was adapted to growth in vitro by passage in upright 75-cm² plastic culture flasks (Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, Calif.) in RPMI 1640 with 20% fetal calf serum (FCS) (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), glutamine (2 mM), penicillin (200 U/ml), and streptomycin (200 U/ml). An adherent variant of P388 was selected for at passage 7 by culture for 14 d at high cell densities in flat 75-cm² culture flasks. Spent media containing the nonadherent cells was removed twice weekly and replaced with fresh media. After an additional 2-wk culture period, the adherent cells were then passed twice weekly by vigorous rinsing of the adherent monolayers with 10 rinses of phosphate-buffered saline (PBS), followed by removal of the adherent cells with trypsin (0.25%) and EDTA (0.02%). Cloned cell lines derived from the adherent and nonadherent forms of P388 were established by limiting dilution techniques in 96-well flat-bottomed tissue culture plates. Cloning medium for the nonadherent form of P388 consisted of RPMI 1640 with 20% FCS; adherent clones were cultured in the same medium supplemented with 10% P388 conditioned medium (CM). Routine maintenance of the adherent clones consisted of weekly subculturing after removal of adherent cells with EDTA (0.1%). Once clones had been established, they were maintained in RPMI 1640 containing 10% FCS and 5% CM.

Preparation of CM. 1 × 10⁶ P388 cells were seeded into an upright 75-cm² culture flask containing 25 ml of RPMI 1640 plus 10% FCS. After incubation at 37°C for 7 d, cells were removed by filtration through Whatman no. 2 filter paper. The pH of the supernatant was adjusted to 7.2 with 0.1 N NaOH and then filter sterilized through a 0.2-μm filter (Nalgene, Rochester, N. Y.) before use as CM.

Assay for Fc and C3b Receptors. Sheep erythrocytes (E) in Alsever's solution were washed in RPMI 1640, and 1 × 10⁹ to 5 × 10⁹ cells were incubated at 37°C for 30 min in subagglutinating amounts of either rabbit anti-E IgM or IgG (Cordis Laboratories Inc., Miami, Fla.) in 0.2 ml of RPMI 1640. After two washes in RPMI 1640, IgG-coated E [E(IgG)] were suspended to 1 × 10⁹ E/ml for use in the Fc receptor (FcR) assay. E(IgM) were incubated an additional 10 min in a 1:10 dilution of fresh frozen DBA/2 mouse serum as a source of complement. After washing, these erythrocytes [E(IgM)C3b] were used as an indicator for C3b receptors (C3bR). The assay for FcR or C3bR consisted of incubating 5 × 10⁴ to 10 × 10⁴ cells in either a 2-cm² tissue culture well (adherent clones) or in a 12 × 75-cm glass tube (nonadherent clones) in 0.5 ml RPMI 1640 with 5 × 10⁸ of the appropriate indicator erythrocytes for 60 minutes at 37°C. Adherent cell lines were rinsed twice in PBS and were scored visually for E binding; nonadherent cell lines were scored directly in a hemocytometer. Any cell that bound three or more E was scored as positive. Controls for positive E-rosette binding consisted of resident peritoneal macrophages that were >95% positive for FcR and C3bR.

Phagocytosis. Adherent cell lines were assayed for phagocytosis of E and E(IgG) in an assay that was identical to the FcR and C3bR assay except that erythrocytes were lysed by a 60-s incubation in Tris-buffered ammonium chloride (0.83%). Phagocytosis-positive cells were determined as any cell internalizing one or more erythrocytes. Latex phagocytosis was determined in a similar manner; 5–10 × 10⁴ adherent cells were incubated for 60 min in 2-cm² wells with 1 × 10⁹ latex spheres (0.81 μm diameter, Difco Laboratories, Detroit, Mich.) in 0.5 ml of RPMI 1640 plus 10% FCS.

Staining Procedures. The nonspecific esterase stain was performed by the method of Li et al. (10) using α-napthyl-butyrate as substrate. Peroxidase reactivity was determined by the method of Kaplow (11). Differential staining was determined by a modified Wright's stain: air-dried smears were stained for 60 s in a solution containing eosin Y (0.18%) and methylene blue

(0.07%) in absolute methanol and counter-stained for 90 s in a solution containing methylene blue (0.14%) and azure II (0.12%) in phosphate-buffered distilled water, pH 6.3.

Immunofluorescence. The expression of several cell surface antigens was determined by indirect immunofluorescence. The following alloantisera were utilized: (SJL × B10) anti-B10.S(24R) (anti-H-2D^d) (gift of Dr. D. C. Shreffler, Washington University, St. Louis, Mo.), (B10.LG × A.TFR-4) anti-B10.D2 (anti-H-2I^d), (B10 × A) anti-B10.D2 (anti-I-A.11,16), and (A.CA × B10.HTP) anti-A.TL (anti-I-A.2) (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Md.). The following hybridoma cell lines were obtained from the Salk Institute: 30H12 (anti-Thy-1.2), M1/70.15 (anti-Mac-1). All hybridoma products used were precipitated with 50% NH$_4$SO$_4$ from culture supernatant, dialyzed against 100 vol of PBS (pH 7.8), and stored at −20°C before use. Fluorescein-conjugated antisera were either F(ab')$_2$ fragments of rabbit anti–mouse IgG (heavy- and light-chain specific) or F(ab')$_2$ fragments of rabbit anti–rat IgG (heavy- and light-chain specific) (N. L. Cappel Laboratories Inc., Cochranville, Pa.). Anti-theta and anti–mouse brain antigen reagents were obtained from Litton Bionetics (Kensington, Md.). All antisera were diluted as needed in RPMI 1640 plus 5% FCS and 0.1% sodium azide.

Adherent cell lines were removed with 0.1% EDTA in RPMI 1640. Adherent and nonadherent cell lines were washed twice at 4°C in RPMI 1640 containing 5% FCS and 0.1% sodium azide. 5 × 10^6 cells were resuspended in 10 × 75-cm glass tubes in 0.2 ml of a 1:10 dilution of the first antibody. After a 30-min incubation at 4°C, the cells were washed three times with 3 ml of RPMI 1640 and resuspended in 0.2 ml of the fluoresceinated second antibody (1:10) for 30 min at 4°C. Cells were washed an additional three times and then resuspended in 0.2 ml of medium for viewing in the microscope or to 1 × 10^6 cells/ml for analysis by flow microfluorometry on an EPICS IV cell analyzer (Coulter Electronics Inc., Hialeah, Fla.). 10^3 cells were analyzed for each determination for forward angle light scatter and peak green fluorescence. Nonviable cells were gated out by light scatter before analysis of fluorescence. Visual determination of percent fluorescence was made on 200 cells per determination. Nonspecific fluorescence was evaluated with cells stained only by the fluoresceinated second antibody and with normal mouse serum as the primary antiserum.

Absorptions of antisera were performed in 1.5-ml polypropylene conical tubes (Eppendorf; Brinkmann Instruments, Inc., Westbury, N. Y.) containing either 10^5-10^8 tumor cells or nylon wool–adherent splenic B cell enriched fractions and 0.1 ml of a dilution of the appropriate antisera. After a 30-min incubation at 4°C, the cells were pelleted by centrifugation and the absorbed antisera were immediately assayed for reactivity by either immunofluorescence or complement-mediated cytotoxicity (CMC).

CMC. The procedure for CMC was essentially the same as for immunofluorescence except that rabbit complement was used rather than a fluoresceinated second antibody. Freshly reconstituted rabbit complement (low-tox-M; Cedarlane Laboratories, Hornby, Ontario, Canada) was preabsorbed for 30 min at 4°C just before use with C3H/HeN spleen cells (10^8 cells/ml of complement) Complement was then diluted 1:10 in RPMI 1640 plus 5% FCS and 0.1% sodium azide; 0.1 ml was then added to each appropriate cell pellet. Tubes were incubated for 30 min at 37°C and then immediately placed on ice. 0.1 ml of trypan blue in PBS (1.0%) was added to each tube 5 min before microscopic determination of percent cytotoxicity (200 cells per determination).

T Cell Proliferation Assay. Asceptically removed mouse speens were pressed through a sterile 100-mesh stainless steel grid. The single-cell suspension was washed twice by centrifugation (150 g, 5 min) in RPMI 1640 plus 5% FCS and 2-mercaptoethanol (5 × 10^{-5} M). Spleen cells were enriched for T cells by the method of Julius et al. (12) on nylon wool columns. T cells (2 × 10^5 to 3 × 10^5) were mixed with varying ratios of mitomycin C–treated stimulator cells in 96-well flat-bottom tissue culture plates in 0.2 ml of RPMI 1640 containing 5% FCS and 2-mercaptoethanol (5 × 10^{-5} M). Plates were cultured for various periods of time. 50 μl of fresh medium containing 2.0 μCi methyl-[^{3}H]thymidine (sp act 2 C/mM; Schwarz/Mann Div, Becton, Dickinson & Co., Orangeburg, N. Y.) was added to each well 18 h before cell harvest. Cultures were collected onto glass-fiber filters by a semiautomatic cell harvester (Otto Hiller Co., Madison, Wisc.). Incorporated radioactivity was measured by liquid scintillation counting in a Beckman counter, model LS-330.

Assay for Cytolytic T Lymphocytes (CTL).   Induction of CTL was performed in upright 25-cm² tissue culture flasks containing 2 × 10⁷ nylon wool–enriched splenic T cells and either 2 × 10⁷ mitomycin C–treated spleen cells or 6.7 × 10⁶ mitomycin C–treated P388 clones in 10 ml of RPMI 1640 containing 5% FCS and 2-mercaptoethanol (5 × 10⁻⁵ M). After 5 d of incubation at 37°C, the cells were washed once and used as effector cells in a ⁵¹Cr release assay. To prepare lipopolysaccharide (LPS) blast cell targets, 5 × 10⁷ nucleated spleen cells were cultured in 10 ml of RPMI 1640, 5% FCS containing 10 μg/ml of LPS (*Salmonella typhosa* 0901; Difco Laboratories Inc.). All target cells were labeled with ⁵¹Cr by culturing 2 × 10⁶ cells in 0.2 ml of Tris-buffered minimum essential medium, pH 6.8, containing 100 μCi of ⁵¹NaCrO₄ (New England Nuclear, Boston, Mass.) for 30 min at 37°C. Varying numbers of effectors (0.1 ml) were seeded in triplicate into round-bottomed microtest plates (Flow Laboratories, Linbro Div., Hamden, Conn.). 1 × 10⁴ ⁵¹Cr-labeled target cells were added to each well. The plates were centrifuged for 30 s at 200 g and then incubated for 4 h at 37°C. Plates were centrifuged again at 20 g for 5 min, and 0.1 ml of the supernatant was removed for counting in a gamma radiation detector (Nuclear Chicago Corp., Chicago, Ill.). The percent specific release from the target cells was determined by the formula: percent specific release = (experimental release − spontaneous release)/(total release − spontaneous release) × 100.

Results

Isolation and Surface Characterizations of Clones of P388 Leukemia.   The P388 leukemia cell line is routinely maintained by in vivo passage intraperitoneally in an ascites form. After adaptation by 7 in vitro passages in upright 75-cm² flasks in medium consisting of RPMI 1640 plus 20% FCS, P388 was cloned in 96-well, flat-bottomed tissue culture plates by limiting dilution techniques to an average density of 1.0 or 0.5 cells/well. After allowing for settling, wells were individually examined microscopically for the presence of single cells. 24 clones were established by this technique, all of which grew as nonadherent cells. At the same time that P388 was cloned for nonadherent lines, it was also selected for growth in the adherent form by culture at passage 7 in 75-cm² flasks in the flat position so that the tissue culture prepared surface was exposed to the cells. At passage 9, numerous adherent cells were seen, and the nonadherent cells were subsequently removed by 10 vigorous washes of the flask in PBS; the remaining adherent cells were refed and cultured. The rinsing and reculturing procedure was repeated twice-weekly for 2 wk and at passage 11, the adherent form of P388 was also cloned by limiting dilution in RPMI 1640 plus 20% FCS with 10% CM, which was required for outgrowth of the adherent clones. Eight clones were isolated by these procedures; all grew as adherent monolayers. The morphological characteristics of the adherent and nonadherent clones can be seen in a fixed, stained preparation in Fig. 1. The nonadherent clones of P388 had a uniform spherical shape with a large nucleus-to-cytoplasm ratio. The cytoplasm was basophilic with few recognizable cytoplasmic elements. The adherent clones displayed a very distinctive dendritic cell–like appearance. The dendritic processes that emanated from these clones varied tremendously from cell to cell. Very broad to very thin processes extended from the cell surfaces, often displaying extensive secondary and tertiary branching characterized by tubular or bulbous protrusions and/or large, thin veils of cytoplasm. The cytoplasm appeared less basophilic than in the nonadherent clones, and many phase-dense granules were apparent. Nuclei in the adherent clones displayed variable shapes, apparently in relationship to the variable cell shape; nucleoli numbering two to three per cell were prominent in the adherent clones.

All eight of the adherent clones and several of the nonadherent clones were phenotyped for the presence of several cell surface antigens, surface receptors, and
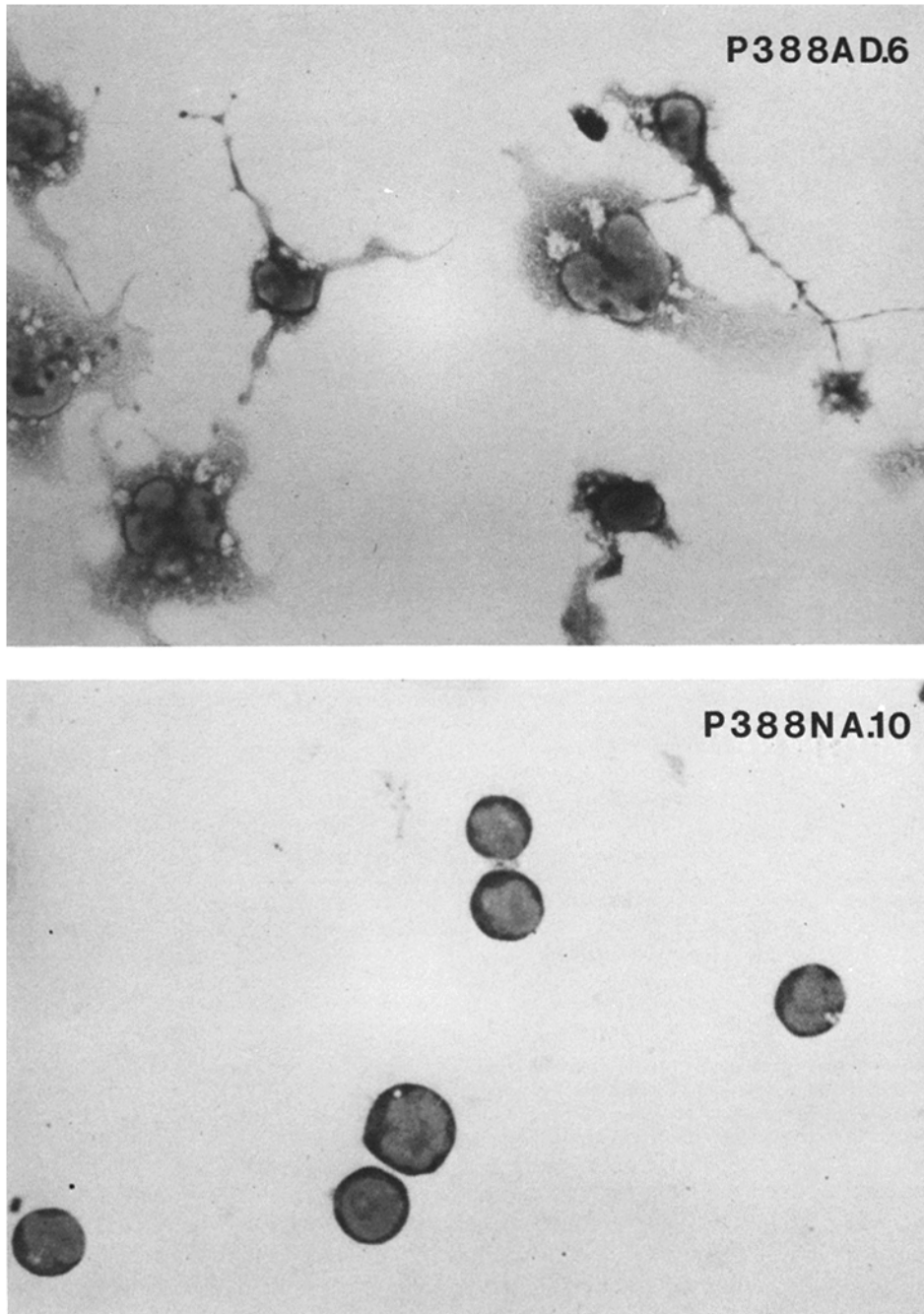
FIG. 1. Morphology of an adherent (P388AD.6) and nonadherent (P388NA.10) clone of P388. 24-h cultures were fixed and stained.

TABLE I

Cell Characteristics of Adherent and Nonadherent Clones of P388*

Characteristic	Adherent P388	Nonadherent P388
	%	
Surface Antigens‡		
Ia	24–69	13–42
H-2D	>95	>95
Mac-1	>95	0
Thy-1	>95	>95
sIg	0	0
Receptors§		
FcR	0.5–7.3	2.5–11.0
C3bR	0.0–2.0	0.0–2.5
Enzymes‖		
Peroxidase	0	0
Esterase	Moderate	Weak
Functions¶		
Phagocytosis	0	0

* Values expressed are the ranges of expression for the indicated characteristics for at least five representative clones per assay. Where single values are expressed, all clones tested gave equivalent values.

‡ Negative controls were stained with normal mouse serum as the primary antiserum and were stained only with the fluoresceinated second antiserum. Values for negative controls were consistently <2%.

§ Values for positive controls (resident peritoneal DBA/2 MΦ) were >95%.

‖ Positive controls for peroxidase were murine peripheral blood monocytes and for nonspecific esterase were resident peritoneal MΦ.

¶ Resident peritoneal MΦ were the positive controls for phagocytosis of latex and E(IgG).

TABLE II

Expression of Surface Ia on Clones of P388*

Clone	AD.1	AD.2	AD.3	AD.4	AD.6	P388AD‡
% Positive	32	55	24	49	50	69

Clone	NA.3	NA.4	NA.6	NA.17	NA.19	P388p§
% Positive	39	34	42	31	15	13

* Values represent the percentage of cells within the indicated clones that demonstrated positive immunofluorescence. Negative controls for each clone consisted of a cell preparation stained with normal mouse serum and the fluoresceinated second antibody and was <2% positive.

‡ P388AD refers to the uncloned adherent form of the P388.

§ P388p refers to the tissue culture passaged P388 leukemia.

cytoplasmic enzymes. These data are represented in Table I. The expression of surface H-2I- and H-2D-region gene products as well as MΦ-associated (Mac-1) and T cell-associated (Thy-1.2) antigens was determined by indirect immunofluorescence. All clones, whether adherent or nonadherent, were positive for the expression of Ia antigens; the intensity of staining varied for individual cells within any clone, but the percent positive fraction for individual clones remained relatively constant from experiment to experiment. The adherent clones generally had a moderately higher fraction of Ia-positive cells than the nonadherent clones; for example, in a representative experiment, the adherent clones ranged from 24 to 69% positive, whereas the

nonadherent clones were 13 to 42% positive using alloantiserum directed against the entire I region (Table II). No difference in the relative intensity of Ia expression was apparent by visual observation or analysis on the EPICS IV cell analyzer between adherent and nonadherent clones when an alloantiserum directed against the entire I region was utilized, (B10.LG × A.TFR-4)-anti-B10.D2. All clones strongly expressed H-2D antigens (>95%). In contrast to I region gene products, all cells in any one clone equally expressed D region gene products, and the intensity of Ab staining against H-2D was substantially stronger than for H-2I. A clear difference between adherent and nonadherent clones was seen in the presence of Mac-1 antigen. All of the adherent clones had surface Mac-1 antigen (equally as strong as H-2D antigen); however, no expression of this antigen was detectable in the nonadherent clones. In contrast to the MΦ-associated antigens, all clones bound monoclonal anti-Thy-1.2 antibody to a moderate and variable extent. This was verified with an alloantiserum and a monoclonal reagent directed against Thy-1.2 as well as a rabbit anti–mouse brain antigen reagent. Surface immunoglobulin (Ig) was not found in any of the clones as determined by direct immunofluorescence. In addition, clones of P388 possessed low or nondetectable levels of receptors for the Fc portion of Ig or for the C3b fragment of C3. All clones were negative for cytoplasmic peroxidase; however, some staining for nonspecific esterase was detected and this was somewhat stronger in the adherent clones. Phagocytic activity was assayed in the clones with SRBC, opsonized SRBC, and latex particles; uptake of particles has never been seen in either type of P388 clones.

The expression of surface Ia was quantitated visually and by flow microfluorometry (FMF). The computer-generated data in Fig. 2 are from a representative experiment
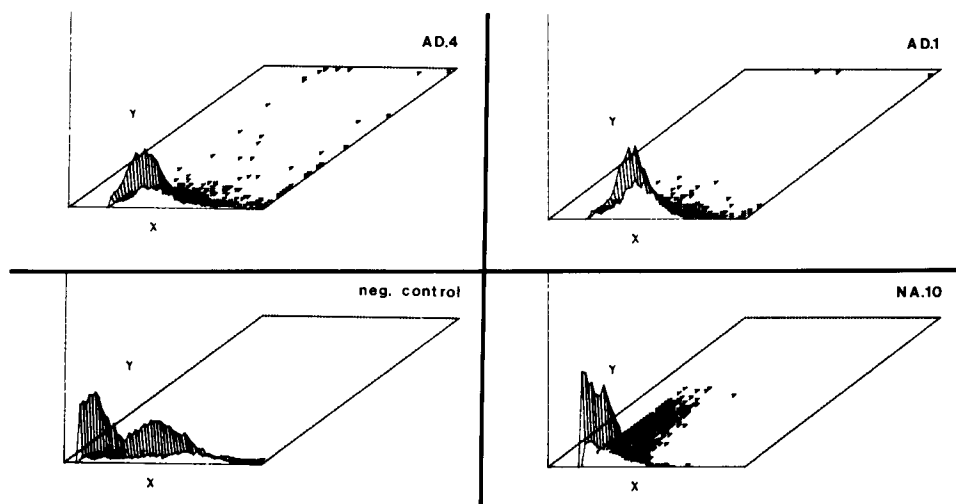

FIG. 2.   Expression of Ia antigens by clones of P388 leukemia. Adherent clones (AD.4 and AD.1) and the nonadherent clone (NA.10) were analyzed by flow microfluorometry for the presence of surface Ia after indirect immunofluorescent staining. Cells were analyzed simultaneously for light scatter and peak green fluorescence after gating out the nonviable cell fraction. x-Axis represents relative cell size (light scatter); y-axis represents relative intensity of fluorescence; height of peak represents cell number. Negative control was an adherent clone stained with normal mouse serum and the fluoresceinated second antibody. Percent fluorescence for each group; AD.4, 46%; AD.1, 51%; NA.10, 44%; neg. control, 2%.
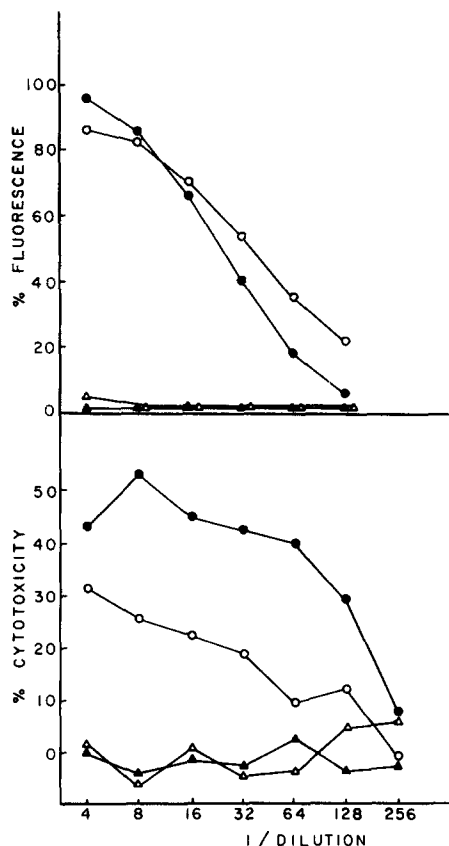
FIG. 3. Binding of anti-I-A.11,16 but not anti-IA.2 alloantiserum to P388 clones. Immunofluorescent analysis of an adherent P388 clone (AD.2) and a nonadherent clone (NA.17) with an appropriate (anti-IA.11,16) and inapppropriate (anti-IA.2) reagent (upper panel). The second reagent was fluoresceinated rabbit anti-mouse IgG F(ab)$_2$ fragments. In the lower panel the specificity of the reagents was evaluated by complement-dependent cytotoxic activity against DBA and C3H enriched splenic B cells. Upper panel: (●) NA.17/IA.11,16; (▲) NA.17/IA.2; (○) AD.2/IA.11,16; (△) AD2/IA.2. Lower panel: (●) DBA/IA.11,16; (▲) DBA/IA.2; (○) C3H/IA.2; (△) C3H/IA.11,16.

in which FMF was utilized to quantitate surface Ia by indirect immunofluorescence on several of the P388 clones. An alloantiserum against the entire I region was utilized in these experiments. In the negative control, which was stained with normal mouse serum and the fluoresceinated second antibody, most of the cells were grouped along the plane of the x-axis, indicating very little immunofluorescence. The fraction of cells that was positive in the negative control was only 2% in this experiment as compared with a control cell sample that was not stained with fluorescent reagents. In contrast, both of the adherent clones (AD.4 and AD.1) and the nonadherent clone (NA.10) had cells of varying fluorescent intensity along the y-axis. Although it would appear from the data that clone NA.10 expressed more Ia antigen than did the adherent clones, the actual percentage of Ia⁺ cells in this experiment and others was quite similar among the adherent and nonadherent clones. The fraction of cells positive for immunofluorescence in Fig. 2 was: AD.4, 46%; AD.1, 51%; NA.10, 44%.
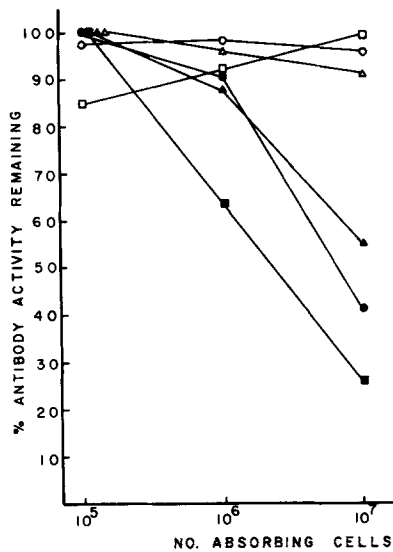
Fig. 4.   Absorptive capacity of P388 clones for an alloantiserum directed against IA.11,16 specific-
ities. 0.1 ml of a 1:6 dilution of either anti-IA.11,16 or anti-IA.2 was absorbed with the indicated
number of cells for 30 min at 4°C. Absorbed anti-IA.11,16 was then assayed for binding to
P388AD.6 by immunofluorescence (closed symbols). Absorbed anti-IA.2 was assayed for antibody
to nucleated C3H enriched splenic B cells by CMC (open symbols). Data is expressed as percent of
the unabsorbed activity remaining postabsorption: unabsorbed anti-IA.11,16 against P388AD.6
yielded 96% positive immunofluorescence; unabsorbed anti-IA.2 against C3H B cells yielded 53%
cytotoxicity. Control experiments indicated that each antiserum could be completely absorbed by
spleen cells only of the appropriate H-2 haplotype. (●) P388/IA.11,16; (○) P388/IA.2; (■) AD.6/
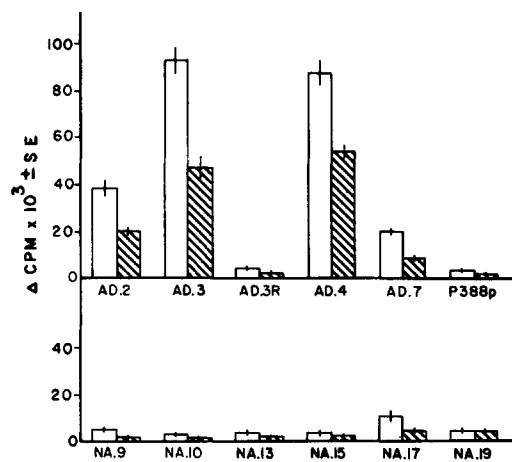IA.11,16; (□) AD.6/IA.2; (▲) NA.10/IA.11,16; (△) NA.10/IA.2.

Fig. 5.   Stimulation of allogeneic T cell proliferation by adherent clones of P388. 3 × 10^5 nylon
wool-enriched C57BL/6 T cells were cultured for 5 d with the indicated mitomycin C-treated
clone at an S:R of 1:30 (open bars) or 1:100 (closed bars). Incorporation of [^3H]thymidine (2 μCi/
well) was determined during the last 18 h of culture. Δcpm is the incorporation in cultures
containing both T cells and stimulators minus the sum of T cell cultures alone and stimulators
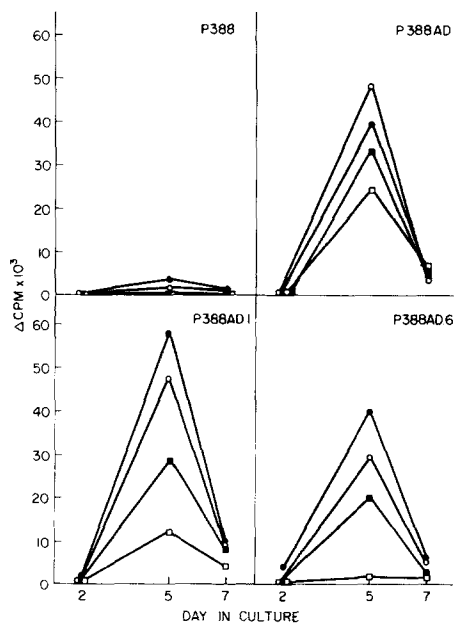alone. Incorporation by T cells alone = 440 ± 5 cpm.

Fig. 6. Kinetics of allogeneic T cell stimulation by adherent P388 clones. 3 × 10⁵ nylon wool-enriched C57BL/6 T cells were cultured for the indicated number of days and at the indicated S:R (●, 1:3; ○, 1:9; ■, 1:27; □, 1:81) with mitomycin C–treated adherent clones (P388AD.1, P388AD.6), uncloned adherent P388 (P388AD) or parental P388 (P388). Δcpm is the incorporation in cultures containing both T cells and stimulators minus the sum of T cell cultures alone and stimulators alone. Incorporation by T cells alone ranged from 809 to 328 cpm during the experiment.

It is clear from these data plots that not only do both adherent and nonadherent forms of P388 express Ia antigens, but there is also a broad range of fluorescent intensity within each respective clone. We are not certain at this time whether cell-to-cell variation in Ia expression represents subpopulations within the clones or cell-cycle-dependent expression of the I region gene products.

Variable expression of Ia has been noted in all clones that have been screened for Ia with the antiserum (B10.LG × A.TFR-4)-anti-B10.D2 (Table II). To verify that the P388 clones express Ia antigens, we performed a second set of experiments using alloantisera with more restricted specificity. Antisera against the subregion specificity I-A.11,16 (H-2^d specific) and I-A.2 (H-2^k specific) were utilized as appropriate and inappropriate reagents. When these antisera were titrated by immunofluorescence against an adherent (P388AD.2) and nonadherent (P388NA.17) clone, only the antiserum reactive with the d haplotype reacted (>80%) with the clones (Fig. 3, upper panel); however, when these same sera were titrated by CMC against DBA/2- or C3H-enriched splenic B cells, each antiserum reacted against the appropriate haplotype (Fig. 3, lower panel). Cell-to-cell variation was also detectable with antisera against I-A subregion antigens, although the intensity of staining was much stronger with this reagent than with the alloantiserum directed against the entire I region. These data indicate that the I-A subregion is strongly expressed in both adherent and nonadherent P388 clones and that percentage of cells that expresses Ia antigens is significantly higher than was detected in previous experiments utilizing an alloanti-
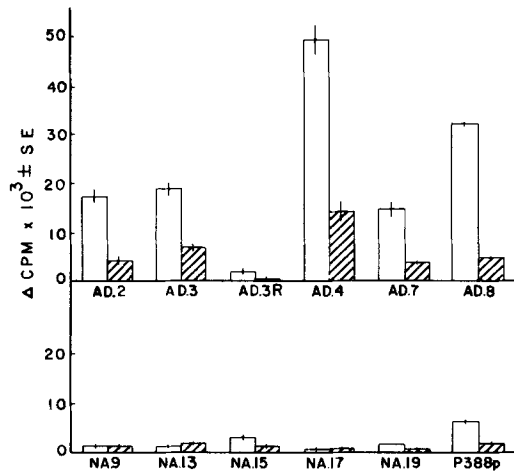
FIG. 7. Stimulation of syngeneic T cell proliferation by adherent clones of P388. 3 × 10^5 nylon wool-enriched DBA/2 T cells were cultured for 5 d with the indicated mitomycin C-treated clone at an S:R of 1:10 (open bars) or 1:30 (closed bars). Incorporation of [^{3}H]thymidine (2 μCi/well) was determined during the last 18 h of culture. Δcpm is the incorporation in cultures containing both T cells and stimulators minus the sum of T cell cultures alone and stimulators alone. Incorporation by T cells alone = 724 ± 50 cpm. P388p refers to the uncloned tissue culture passaged P388 leukemia.
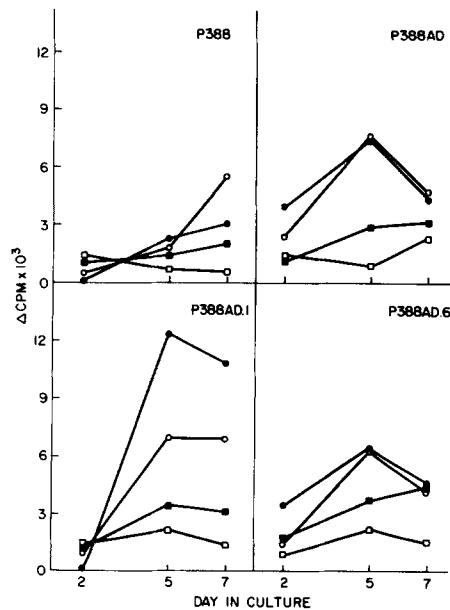

FIG. 8. Kinetics of syngeneic T cell stimulation by adherent P388 clones. 3 × 10^5 nylon wool-enriched DBA/2 T cells were cultured for the indicated number of days and at the indicated S:R (●, 1:3, ○, 1:9; ■, 1:27; □, 1:81) with mitomycin C-treated adherent clones (P388AD.1, P388AD.6), uncloned adherent P388 (P388AD) or parental P388 (P388). Δcpm is the incorporation in cultures containing both T cells and stimulators minus the sum of T cell cultures alone and stimulators alone. Incorporation by T cells only ranged from 1,409 to 816 cpm during the experiment.
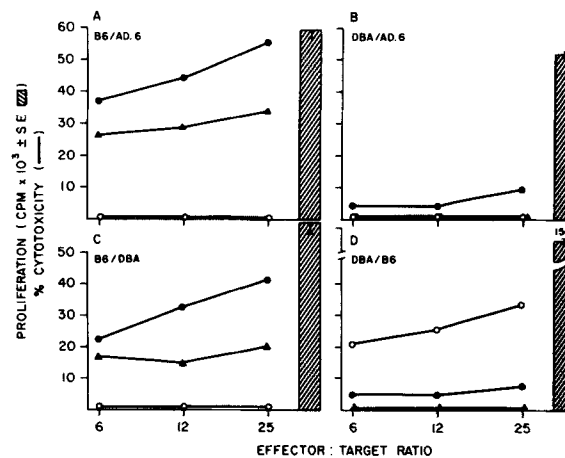
FIG. 9.   Failure to induce cytotoxic T cells during a P388AD-induced SMLR. 2×10^7 nylon wool–enriched splenic T cells from either DBA/2 or C57BL/6 mice were cultured with mitomycin C–treated P388AD.6 (panels A and B) (6.7×10^6) or with mitomycin C–treated DBA/2 (panel C) or C57BL/6 (panel D) unfractionated spleen cells (2×10^7). T cell cultures were simultaneously established in microculture to assay for proliferation as described in Materials and Methods. 5 d after culture at 37°C, the CTL cultures were assayed for cytotoxicity in a 4-h ^{51}Cr release assay against P388AD.6 (●), LPS-stimulated DBA/2 blast cells (▲), or LPS-stimulated C57BL/6 blast cells (○). Spontaneous release was 28% for P388AD.6, 36% for DBA/2 blasts, and 39% for C57BL/6 blasts. T cell proliferation (hatched bars) was determined at day 5 of culture after an 18-h pulse with [³H]thymidine (2 μCi/well). Proliferation by unstimulated T cells was 10,355 cpm for DBA/2 and 3,066 for C57BL/6.

serum directed against the entire I region. Quantitative absorptions were also performed against the same I-A specific antisera with P388 clones or spleen cells as absorbing cells. It is clear in Fig. 4 that both the adherent and the nonadherent clones (P388AD.6 and P388NA.10, respectively) as well as the parent line P388 were able to absorb activity from anti-I-A.11,16 but had no absorptive capacity against anti-I-A.2. In each of three experiments, the adherent clone tested had slightly better absorptive capacity than either the nonadherent clone or parental P388, suggesting that the density of I-A gene products may be greater on clone AD.6.

Induction of Allogeneic and Syngeneic Mixed Lymphocyte Reactions with P388 Clones. Many investigators have demonstrated that differences in Ia antigens can stimulate a strong proliferative response by allogeneic T lymphocytes in a primary mixed lymphocyte response. We have tested the ability of the P388 clones to stimulate proliferation of unprimed C57BL/6 T cells during a 5-d in vitro culture of nylon wool–enriched T cells and mitomycin C–treated P388 clones. All of the adherent clones, except AD.3R, strongly stimulated the proliferation of allogeneic T cells; pronounced stimulation occurred at stimulator:responder ratios (S:R) as low as 1:100 (Fig. 5). In contrast, the nonadherent clones did not induce proliferation at S:R of either 1:30 or 1:100; S:R as high as 1:1 have been tested without significant stimulation (Fig. 5). Clone AD.3R, which did not stimulate T cell proliferation, is a very interesting cell line in that it represents a spontaneous reversion of adherent clone AD.3 to a nonadherent form. This phenotypic variant simultaneously lost its ability to augment T cell proliferation; moreover, it still retained its expression of Ia antigens, but lost the Mac-1 antigen.

The kinetics of allogeneic T cell stimulation as well as the effect of several S:R was determined with the clones AD.1 and AD.6 and with parental P388 and uncloned adherent P388. The data in Fig. 6 indicate that the adherent forms of P388 stimulated C57BL/6 T cells in a dose-related manner at S:R as low as 1:81. The peak stimulation occurred after 5 d in culture and thereafter declined rapidly. Parental P388 failed to stimulate at any S:R ratio or time-point, suggesting that the failure to stimulate was not attributable to suboptimal conditions, but rather to an intrinsic difference in the way that the stimulators interacted with the responding lymphocytes.

Recent data from several laboratories (13–16) have described a syngeneic mixed lymphocyte reaction (SMLR) in which Ia-bearing stimulator cells can induce proliferation of syngeneic T cells in the absence of antigen and have suggested that the SMLR may be a requirement for normal antigen-specific T cell proliferation (17). We have also tested the P388 clones for the ability to stimulate the proliferation of syngeneic DBA/2 T cells under conditions similar to the allogeneic mixed lymphocyte reaction (AMLR) (Fig. 7). Although the magnitude of the syngeneic response was weaker, the exact pattern of response was repeated; i.e., only the adherent clones of P388 were capable of stimulating the proliferation of syngeneic T cells. The kinetics of the SMLR were also determined and are presented in Fig. 8. As was seen for the AMLR, peak stimulation of T cells in the SMLR occurred after 5 d in culture with adherent forms of P388 in a dose-related manner; however, in this experiment, some stimulation by the parental P388 was noted at day 7 of culture. Stimulation by the parental line of P388 was not a consistent finding and is thought to be related to culture conditions of P388 that permit outgrowth of adherent cells in the parent line. It should also be pointed out that conditioned medium prepared from monolayers of the adherent clones had no stimulatory effect on syngeneic T cells, suggesting that cell-to-cell contact may be required for the SMLR (data not presented).

One characteristic of an SMLR is that the proliferative response does not induce CTL (6). To determine whether the proliferation of DBA/2 T cells against clones of P388AD was a true SMLR, we cultured T cells from DBA/2 or C57Bl/6 spleens for 5 d with mitomycin C-treated P388AD.6, DBA/2 spleen cells, or C57Bl/6 spleen cells. Each culture was then assayed for the presence of CTL in a ^{51}Cr release assay against each stimulator type as the target cell. The data presented in Fig. 9 demonstrate that strong proliferative responses occurred with both syngeneic and allogeneic stimulation. However, CTL induction only occurred in cultures with allogeneic stimulators (B6/AD.6, B6/DBA, DBA/B6), and in each case the CTL were specific for the H-2 haplotype of the stimulator cell. In spite of strong proliferation of DBA/2 T cells cultured with P388AD.6, no CTL response was detected either against the tumor line (tumor-specific CTL) or DBA/2 blast cells (H-2^d-specific CTL). DBA/2 T cells were, however, able to generate a CTL response against B6 spleen cells (H-2^b-specific CTL).

Discussion

We have described in this report the isolation of several Ia-bearing adherent cell lines cloned from the P388 leukemia cell line, which are capable of inducing an SMLR. These cell lines possess many morphological and functional similarities to the dendritic cells of the spleen described by Steinman and co-workers (4–6).

A distinctive cellular morphology was seen under phase-contrast microscopy. Under

optimal conditions, nearly all of the cells in culture were flat and many possessed extensive dendritic processes. The cytoplasm appeared to contain relatively few cytoplasmic granules; occasional phase-lucent vesicles were seen along with small phase-dense granules. Electron microscopic analysis indicated an absence of phago-lysosomes and an abundance of mitochondria, many of which were seen lining the length of dendritic processes (data not presented). These dendritic-like cells have been analyzed by microcinematography and appeared to be very active during culture; dendritic processes constantly extended and retracted from the cell surfaces and the nuclei of the cells often roamed throughout the cytoplasm and assumed various shapes. Many cells entered a quiescent period just before cell division in which the cells acquired a more spherical shape.

Adherent clones of P388 expressed only one clear marker normally associated with MΦ; virtually 100% of the cells in each adherent clone strongly expressed the Mac-1 antigen described by Springer et al. (18). These data would suggest that these tumor lines have their lineage in the MΦ-granulocyte series and, if these cells are truly dendritic cell tumors, this also suggests the same lineage for the dendritic cell. However, it must be pointed out that because these are tumor cell lines, transforma-tion-associated gene derepression could be responsible for the expression of Mac-1. For example, the adherent clones of P388 simultaneously express Mac-1 and Thy-1.2 antigens, and it is unlikely that this dual expression is associated with a common cell origin. B-lymphocyte origin is also unlikely, because these clones did not have surface receptors for Fc or C3b and did not express cytoplasmic or surface Ig. These data reflect certain characteristics of immature MΦ; however, these cells had no cytoplas-mic peroxidase activity normally associated with immature MΦ, and the addition of phorbol myristate acetate to adherent P388 clones was unable to induce more mature characteristics in these cells (data not presented).

Potentially, the most interesting surface characteristic of these cell lines is the expression of I region gene products. To our knowledge, this is the first isolation of stable murine adherent cell lines that synthesize and express large quantities of Ia antigens and directly stimulate syngeneic T cell proliferation. Walker and Warner[2] have previously described Ia$^+$ clones derived from the myelomonocytic leukemia WEHI-3. These clones have properties similar to those of P388AD clones in that they are nonphagocytic, have a low density of Fc and C3b receptors, and express I-A subregion gene products. In contrast to P388AD, the MΦ-replacing functions of WEHI-3 clones can be duplicated with culture supernatants from either the Ia$^+$ clones on the Ia$^-$ parent tumor. We have not been able to induce strong T cell proliferation with culture supernatants from either adherent or nonadherent clones or from the parent tumor P388. The importance of finding Ia expression in adherent P388 clones is that Ia-mediated immune functions can be studied using cloned accessory cell populations that are homogeneous. The first indication that these adherent clones may be useful for a detailed study of accessory cell function is the finding that a strong SMLR can be induced by adherent clones of P388. Hausman et al. (17) have recently suggested that the SMLR is a normal outcome of antigen presentation, and studies by Burger and Shevach (19), Shevach et al. (20), and Schwartz et al. (21)

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indicate that recognition of Ia antigens by antigen-reactive T cells is a necessary link in the triggering of T cell proliferation. The adherent clones of P388 appear to induce a true SMLR for several reasons. In contrast to the experiments with an AMLR, cytotoxic T cells were not induced in proliferating SMLR cultures. Even in the AMLR, the cytotoxic response appears to be directed against a normal cell component and not toward potential tumor-associated antigens, because LPS-stimulated blast cells from DBA/2 mice were also effective target cells. Finally, the strength of the proliferative response in the primary SMLR was stronger than would be expected in a primary response against tumor antigens. It should also be pointed out that differences in the Mls locus that existed in the AMLR cultures apparently played little or no role in the induction of proliferation, because, in SMLR cultures in which an Mls difference existed (BALB/c T cells and P388AD stimulators), an additive effect attributable to Mls disparity was not detected (data not presented).

An interesting observation during these studies was that nonadherent clones of P388, which also express surface Ia antigens, were incapable of stimulating either syngeneic or allogeneic T cell proliferation. These findings are relevant to the understanding of Ia-mediated triggering mechanisms. Although it is clear from many laboratories that Ia molecules on accessory cells are necessary for induction of T cell proliferation, these data suggest that Ia expression itself may not be sufficient. Several alternatives are available to explain these results. One possibility may be that the adherent and nonadherent clones express different subregions of the I region and that only the subregion gene products associated with the adherent clones can trigger proliferation. Many investigators have shown that antisera directed against several I region gene products plus complement deleted antigen-presenting functions; however, in the absence of complement only antisera directed toward particular subregion antigens could functionally block antigen presentation with defined antigens (19–24). For example, Niederhuber and Allen (23, 24) have recently shown that only anti-I-J reagents can block in vitro antibody responses against burro erythrocytes. The studies involving antisera directed against the I-A subregion clearly indicate that all clones, adherent and nonadherent, express the 11 and 16 specificities. These data would suggest that the I-A subregion is not functionally important in induction of T cell proliferation by P388AD clones or that factors other then Ia subregion gene products are required, such as interleukin 1 (IL-1) or another I subregion product. Studies by Farrar et al. (25) have pointed to the importance of IL-1 in the initiation of the proliferative response, and it is possible that the major difference in the stimulatory capacity of the clones is in the ability to synthesize IL-1 during interaction with T cells. Alternatively, the nonadherent clones may synthesize inhibitory factors such as prostaglandin E, which inhibit proliferation. A final explanation may be that spatial orientation of the Ia molecules is different in adherent and nonadherent clones, such that the appropriate determinants on the Ia molecule in the nonadherent clones is not recognized by the T cell receptor. Evidence to support the argument that only certain epitopes on the Ia molecule are critical for the induction of T cell proliferation by accessory cells has recently been presented by Burger and Shevach (19).

It is interesting that the well-characterized macrophage-like cell line, P388D1, was also isolated from the same parental tumor line. This cell line is quite different from the adherent clones described in this report. The P388D1 cell line has many more functional similarities to mature MΦ that the dendritic cell–like lines do not possess,

including phagocytosis and antibody-dependent cellular cytotoxicity (26). IL-1 is produced by stimulated cultures of P388D1 (27). However, P388D1 does not express surface Ia, which may account for its inability to present antigen to lymphocytes (28). We have verified by absorption studies that P388D1 cells do not express Ia antigens and in that respect are quite distinct from P388AD and P388NA. This leads to an apparent dilemma concerning the origin of these new Ia$^+$ clones, because they and P388D1 were both derived from a purportedly Ia$^-$ tumor, P388. However, numerous experiments have indicated that the P388 tumor cells that we maintain in our laboratory does express Ia antigens of the H-2^d haplotype. Ia expression in the parent tumor cell line is not related to passage in vitro, because the original tumor, which has always been maintained by in vivo passage, also expresses Ia. It is not clear why the P388 tumor cells from our laboratory expresses Ia, while P388 from other laboratories is Ia$^-$; however, we speculate that derepression of the I region spontaneously and fortuitously occurred in this particular P388 tumor line at an earlier time in its passage. The isolation of the novel Ia$^+$ adherent cell lines from the tumor, which also possess the ability to stimulate an SMLR, may be of value for future study of antigen presentation and the induction of T cell proliferation in specific immune responses.

Summary

Several adherent cell lines that express surface Ia antigens have been cloned from the P388 leukemia. These cell lines express many morphological and functional similarities to dendritic cells of the spleen. Adherent clones express H-2 gene products from the I and D regions, but do not possess Fc or C3b receptors or surface Ig. The cytoplasm of the adherent P388 clones has an abundance of mitochondria but few phagolysosomes. The adherent clones all strongly stimulate the proliferation of syngeneic or allogeneic T lymphocytes in the absence of antigen; however, cytotoxic T cells directed against the stimulators are induced only during allogeneic stimulation. Nonadherent clones of P388, which also express Ia antigens, do not stimulate either allogeneic or syngeneic T cell proliferation. The isolation of Ia-bearing adherent cell lines makes it possible to study Ia-mediated events in both antigen-dependent and independent immune systems using homogeneous accessory cell populations.

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